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ABSTRACT

The lactating mammary gland synthesizes and secretes large amounts of phosphoproteins that mainly are associated with the casein fraction of milk. The free amino acids and inorganic phosphate of blood serve as building materials for casein, and the final product appears in milk as a colloidal-sized particle, the casein micelle. According to our present concept, the biosynthesis of casein occurs in two steps: synthesis of the polypeptide chain, followed by phosphate addition. Phosphate groups are transferred to the nascent casein by a protein kinase localized in the Golgi apparatus. The enzyme uses adenosine 5′-triphosphate as the phosphate donor and requires divalent cations. Neighboring amino acids may be important in determining which serine residues in casein are phosphorylated. This review discusses historical and current research on the phosphorylation of casein.

INTRODUCTION

Since the major proteins of milk, caseins, contain phosphorus, the synthesis and secretion of phosphoproteins are important functions of the lactating mammary gland. Whereas the phosphoprotein nature of casein was recognized 100 yr ago, knowledge of the biochemical mechanism by which phosphate groups are incorporated into casein has eluded investigators until recently. Advances of the past decade have established a plausible pathway for synthesis and phosphorylation of casein and an understanding of the cellular processes involved. This review covers developments leading to current studies concerned with the phosphorylation of casein. Related material can be found in reviews on milk protein synthesis by Barry (3) and Larson and Jorgensen (24) and on phosphoproteins by Taborsky (47).

Casein

Casein is a family of proteins, arbitrarily defined as those phosphoproteins that are precipitated from raw skim milk by acidification to pH 4.6 at 20 C (55). The major components of whole bovine casein are α_{s1}-casein (45%), β-casein (33%), and κ-casein (15%), each of which displays genetic polymorphism. The identification of individual components has depended upon the resolving power of electrophoretic techniques (48). Polyacrylamide gel electrophoresis in the presence of urea has been used to great advantage for separating and identifying caseins and has been particularly valuable in distinguishing genetic variants. During the past 5 yr, the primary structures of the major bovine caseins have been established (16, 32, 33, 35), and caseins can now be defined on the molecular level. For a more complete discussion of casein, the reader is referred to recent reviews by Thompson and Farrell (49), Mercier et al. (34), Swaisgood (46), and Whitney et al. (55).

The nature of the phosphate groups in casein was the subject of considerably controversy during the 1950's when it was postulated that phosphodiester and/or pyrophosphate groups occurred in casein along with monoester groups. This research has been summarized by McKenzie (31). Final resolution of the problem was accomplished by sequence studies in which the position of the phosphate groups in the major bovine caseins was established, and all phosphate groups were shown to be monoesters of serine (16, 32, 33, 35). The primary structures indicate that α_{s1}-casein, β-casein, and κ-casein contain 8, 5, and 1 phosphomonoester groups per monomer, although minor variations of this pattern occur in certain genetic variants (55).

Role of Blood Inorganic Phosphate

Today it generally is accepted that the phosphorus in casein is derived from the inorganic phosphate of blood. However, the origin of phosphorus and amino acids in casein was a prime concern of early investigators interested in lactation. The availability of radioisotopes was an important development which enabled researchers to trace compounds injected into the bloodstream through the mammary gland and into the milk. In 1938, Aten and Hevesy (1) performed the first tracer experiment on lactation to determine the fate of labeled inorganic phosphate injected into the blood stream of lactating goats. Their results, confirmed by other investigators (22, 41), indicated that the inorganic phosphate of blood was taken up by the mammary gland for the synthesis of casein. Barry (2) observed that labeled inorganic phosphate from blood appeared in milk as free orthophosphate in 1.5 to 2 h while ^{32}P-labeled casein appeared in 2 to 2.5 h. These experiments did not rule out the possibility that the inorganic phosphate in the blood might be converted rapidly to an intermediate in another part of the body prior to its uptake by the mammary gland. Therefore, the effect of other organs was eliminated by demonstrating casein synthesis in an excised bovine mammary gland perfused with blood containing labeled phosphate (2). Subsequently, Sundararajan et al. (45) showed that proteins of milk and mammary gland are phosphorylated at a rate comparable to that of liver phosphoproteins, which have a high rate of phosphorus turnover. These investigators characterized ^{32}P-labeled casein from the milk of a rabbit injected with labeled phosphate and concluded that the high specific activity represented a true incorporation of ^{32}P into the protein. Furthermore, the ^{32}P was present as phosphoserine, and the phosphorus atoms of casein were labeled evenly.

Pathway of Phosphate Incorporation into Casein

When it was established that mammary gland utilizes the inorganic phosphate from blood for the formation of casein phosphorus and that phosphorus incorporation occurs at a rapid rate, attention was focused on the pathway in this conversion. Prior to the elaboration of the genetic code and the current concept of protein synthesis, it was theorized that phosphoserine might serve as an intermediate in casein synthesis. However, no free phosphoserine has been detected in the lactating mammary gland (26), nor are there phosphokinases capable of phosphorylating serine (43). Although phosphoserine can be formed from glucose by the transamination of phosphohydroxypyruvate, there is no support for this mechanism in the mammary gland (25). Phosphopeptides also were considered as intermediates in the synthesis of casein by several investigators who noted phosphopeptides in the lactating mammary glands of rabbits (39) and rats (38). Experiments with isotopes indicated that the mammary peptides showed a high turnover of ^{32}P, which was comparable to the turnover rate of phosphoproteins in milk and mammary gland (39). This high metabolic activity suggested that phosphopeptides could be utilized in casein synthesis. However, these investigators did not rule out the possibility that phosphorylation might occur at the protein level.

To determine whether casein is phosphorylated before or after the completion of the polypeptide chain, Singh et al. (42) investigated protein synthesis in rat mammary gland slices. They studied the effect of puromycin on the incorporation of ^{14}C-labeled leucine and ^{32}P-labeled phosphate into protein as a function of time (Fig. 1). For the first 30 min puromycin inhibited the incorporation of leucine carbon into protein by 80% whereas the incorporation of ^{32}P into protein was unaffected. Since the synthesis of new protein is blocked by puromycin, these investigators concluded that a pool of unphosphorylated protein in mammary gland cells must sustain the phosphorylation during the initial 30 min period. Similar results were obtained by Turkington and Topper (51), who investigated protein synthesis in mouse mammary explants. They provided evidence that the protein phosphorylated in their system was casein and concluded that the polypeptide chain of casein was synthesized before the phosphate groups were added. The concept that unphosphorylated casein is the intermediate which serves as the acceptor for phosphate represented a significant advance in our knowledge of the biosynthesis of casein. Efforts then were directed toward finding enzymes that could phosphorylate the nascent casein.

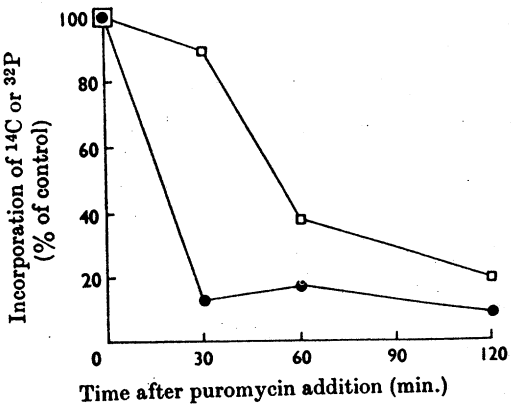

FIG. 1. Effect of puromycin on the incorporation of ^{14}C-lysine (•) and ^{32}P-phosphate (□) into protein by rat mammary gland slices as a function of time. Radioactivity incorporated in the presence of puromycin is expressed as percentage of that incorporated in control experiments where puromycin was not added. Reproduced from Singh et al. (42) with the permission of Biochemical Journal.

Protein Kinases

The first direct evidence for the enzymatic phosphorylation of a protein substrate was provided by Burnett and Kennedy (10) in 1954. Their elegant study of protein kinase (EC 2.7.1.37; ATP:protein phosphotransferase) in rat liver mitochondria clearly defined the enzyme catalyzed reaction as:

$$\text{ATP} + \text{Protein} \xrightarrow{Mg^{2+}} \text{ADP} + \text{Phosphoprotein}.$$

They showed that ATP is derived from inorganic phosphate through oxidative phosphorylation in the mitochondria and that Mg^{2+} is required for protein phosphorylation. Of the various protein substrates tested, only casein was phosphorylated by their enzyme. α-Casein (a mixture of α_{s1}-casein and κ-casein) was phosphorylated at four times the rate of β-casein, and when the casein was dephosphorylated with alkali, phosphate incorporation was reduced drastically.

Several investigators looked for a similar enzyme in mammary gland extracts with the expectation that a protein kinase was involved in the biosynthesis of casein. Although protein kinases were found in mammary tissue, early results were inconclusive. Washed particulate fractions from rabbits incorporated ^{32}P into mammary phosphoproteins (40), and a rat mammary particulate enzyme phosphorylated both casein and dephosphorylated casein (43). However, the metabolic function of these enzymes is not clear. Sundararajan et al. (44) partially purified a protein kinase from the mammary gland of lactating rabbits that required intact sulfhydryl groups as well as ATP and Mg^{2+} for activity. Two phosphoproteins, casein and phosvitin, could be phosphorylated by this enzyme. The phosphorus uptake by casein increased after partial dephosphorylation and decreased after complete dephosphorylation. Because enzymatically dephosphorylated casein was a poorer substrate than native casein, these investigators had difficulty explaining the role of their protein kinase in casein synthesis. The interpretation of many of these early experiments was hampered by a lack of knowledge of the nature and diversity of protein kinases. It now has become apparent that mammalian tissues contain many protein kinases that are involved in cellular activities. Therefore, the problem of distinguishing cellular protein kinases from those involved in casein synthesis was not appreciated fully.

Recent Developments in Protein Kinase Research

Research on protein kinases has expanded greatly since 1968 when Walsh et al. (54) observed that certain protein kinases are activated by cyclic AMP (cyclic adenosine 3′:5′-monophosphate). Since then, many of the investigations have been concerned with cyclic AMP-dependent protein kinases, which generally are involved in control mechanisms mediated through hormones. Less is known about a second category, cyclic AMP-independent protein kinases. Traugh et al. (50) have devised methods for classifying protein kinases based on whether or not they are activated by cyclic AMP. Two excellent reviews (36, 53) summarize current developments in protein kinase research.

The recent expansion of protein kinase research has stimulated investigations of protein kinases in the mammary gland. Like other tissues, the mammary gland shows a multiplicity of protein kinases whose physiological functions have not been clarified. Majumder and Turkington (27, 28, 29) found two protein kinases, one of which was stimulated by cyclic AMP, in the cytosol of the mammary glands of mice and rats. Both enzymes showed a high specificity for histone but exhibited little activi-

ty toward casein; the protein kinases also phosphorylated ribosomal and membrane proteins. These investigators were interested in hormone-dependent phosphorylations and made no attempt to examine the biosynthetic mechanism for the phosphorylation of casein. Waddy and Mackinlay (52) purified protein kinases from soluble extracts of lactating bovine mammary glands. Although these enzymes were purified on the basis of their ability to phosphorylate casein, they were similar to the protein kinases characterized by Majumder and Turkington in that they showed a preference for basic proteins such as histones as phosphate acceptors.

A cyclic AMP-independent protein kinase (kinase C) with a preference for dephosphorylated α_{s1}-casein was extracted from a particulate fraction of bovine mammary gland homogenates by Chew and Mackinlay (13). The dephosphorylated forms of both α_{s1}-casein and a phosphate-rich peptide prepared from α_{s1}-casein by cyanogen bromide cleavage were good substrates, indicating that kinase C could phosphorylate serine residues that normally are phosphorylated in vivo. The phosphorylation occurred in one or more of the serine residues in positions 64 to 68 of the α_{s1}-casein molecule (Fig. 2). However, dephosphorylated β-casein showed no phosphate incorporation by kinase C despite the fact that β-casein has a cluster of serine residues, almost identical to residues 64 to 70 in α_{s1}-casein. Further characterization of

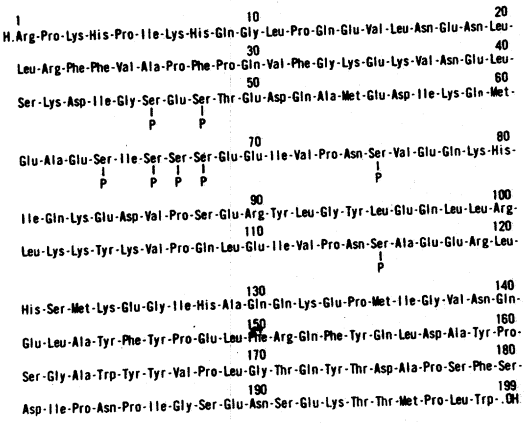

FIG. 2. The primary structure of bovine α_{s1}-casein B according to Mercier et al. (33) and Grosclaude et al. (16).

kinase C will be required to clarify its role in casein synthesis.

Subcellular Location of Casein Kinase

Considerable research has been directed toward understanding the relationship of cell structure to function in lactating mammary tissue. Reviews by Saacke and Heald (37) and Keenan et al. (19) summarize research on the ultrastructure of mammary secretory cells. The biosynthesis of casein occurs on the polyribosomes bound to the membranes of the endoplasmic reticulum (4, 14). It generally is believed that the nascent casein leaves the polyribosomes on the outer surface of the endoplasmic reticulum, enters the intercisternal space, and then is transported to the Golgi region where aggregation of the peptide chains into casein micelles occurs prior to secretion. Electron microscope studies have indicated that casein micelles are formed in the Golgi vesicles by the condensation of protein fibrils (5, 12). It seems logical to postulate that phosphorylation of casein occurs along this pathway. Bingham et al. (8), examining protein kinase activity in subcellular fractions from the lactating mammary gland of rats, provided the first direct evidence that casein is phosphorylated in the Golgi apparatus. Protein kinase from the Golgi apparatus incorporated phosphate into dephosphorylated α_{s1}-casein at more than 10 times the rate for protein kinases in other subcellular fractions (Table 1). The Golgi protein kinase showed a preference for dephosphorylated α_{s1}-casein, which was phosphorylated at a rate significantly higher than that of native α_{s1}-casein. These results support the concept that one of the functions of the Golgi apparatus is to process newly synthesized protein. In the mammary gland, phosphate groups are added to the nascent casein by membrane enzymes of the Golgi apparatus.

Properties of Casein Kinase

The casein kinase from the rat Golgi apparatus is a cyclic AMP-independent protein kinase, which catalyzes phosphate incorporation into casein using ATP as the phosphate donor (7). Dephosphorylated α_{s1}-, β-, and κ-caseins are the best substrates for casein kinase and are phosphorylated at much higher rates than the native caseins (Table 2). Histones, phosvitin, and the

TABLE 1. Subcellular distribution of protein kinase from lactating mammary gland[a].

Fraction	Protein kinase activity	
	α_{s1}-casein	Dephosphorylated α_{s1}-casein
	(pmoles/mg protein/20 min)	
Total homogenate	.16	.20
Golgi apparatus	5.44	24.02
Nuclei	.54	.48
Mitochondria	.54	1.10
Microsomes	1.72	1.80
Cytoplasm	.50	.22

[a]Data taken from Bingham et al. (8); reproduced with the permission of the Journal of Biological Chemistry.

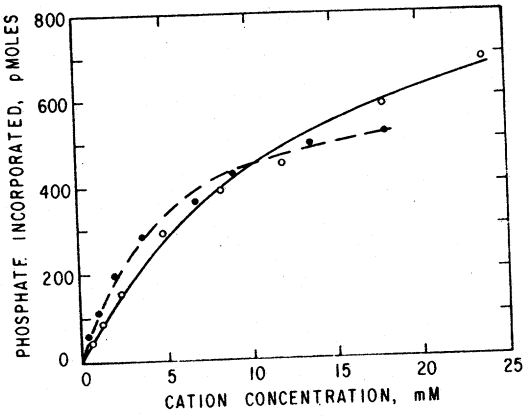

FIG. 3. Effect of cation concentration on casein kinase activity. Phosphate incorporation was measured in the presence of Mg^{2+} (●--●) and in the presence of Ca^{2+} (○—○). Reproduced from Bingham and Farrell (7).

major proteins of whey (β-lactoglobulin and α-lactalbumin) are not phosphorylated appreciably. Studies of divalent metal ion requirements indicate that either Ca^{2+} or Mg^{2+} is essential for catalysis (Fig. 3). Since most other bovine protein kinases are inhibited by Ca^{2+} (23), activation by this cation is an unusual feature.

It is interesting to speculate as to why this casein kinase been so elusive. Inasmuch as the mammary gland secretes large quantities of phosphoproteins, casein kinase should be an abundant enzyme. Majumder and Turkington (28) pointed out that 98% of the total mammary protein kinase is in the cytoplasm. Our calculations (unpublished) show that 80% of the total protein kinase is soluble. Thus, large quantities of soluble protein kinases must obscure the enzyme responsible for the phosphorylation of casein. It is possible that a small amount of enzyme attached to the Golgi membranes could function as a potent immobilized catalyst, phosphorylating casein as it passes through the endomembrane system of the lactating cell.

TABLE 2. Phosphorylation of proteins by casein kinase[a].

Protein substrate	Rate of phosphate addition nmol/mg per 20 min
α_{s1}-casein	8.6
Dephosphorylated α_{s1}-casein	43.0
β-casein	2.5
Dephosphorylated β-casein	32.4
κ-casein	1.3
Dephosphorylated κ-casein	8.5
β-Lactoglobulin	2.1
α-Lactalbumin	1.1
Histone-arginine rich	.1
Histone-lysine rich	.1
Phosvitin	.3

[a]Data taken from Bingham and Farrell (7); reproduced with the permission of the Journal of Biological Chemistry.

Mechanism of Casein Phosphorylation

The fact that dephosphorylated caseins are phosphorylated by casein kinase faster than are native caseins (7, 8) suggests that the enzyme may be incorporating phosphate into the casein at one or more of the sites that originally were phosphorylated. However, quantitative data on the number of serines phosphorylated by casein kinase and the exact location of the phosphorylated sites are lacking.

Mercier et al. (34) proposed a theory for the specificity of casein kinase based on examination of the sequence data of the three main bovine caseins. α_{s1}-Casein B (Fig. 2) has 16 serine residues, eight of which are phosphorylated (33). Every phosphorylated serine in this molecule has a glutamic acid or a phosphoserine

two residues to the right in the sequence, but this configuration does not occur when the serines are phosphate-free. The α_{s1}-casein D variant contains an additional phosphate group, phosphothreonine, which replaces alanine 53; there is a glutamic acid residue two residues to the right of this phosphorylated site (Table 3). Thus, casein kinase recognizes a potential phosphorylation site corresponding to the tripeptide sequence Ser/Thr-X-Glu/SerP, where X is any amino acid. The six genetic forms of β-casein (A^1, A^2, A^3, B, C, and E) also support this hypothesis (17, 35).

This hypothesis explains the phosphorylation of serine residues in α_{s1}- and β-caseins. However, other milk proteins contain potential sites for phosphorylation that do not conform to the proposed theory. β-Lactoglobulin (9) has three potential sites; yet the molecule contains no phosphate. It is possible that the tertiary structure of this molecule interferes with phosphorylation. There is support for this idea from research on lysozyme; cyclic AMP-dependent protein kinases can phosphorylate denatured lysozyme but not native lysozyme with its disulfide bonds intact (11). κ-Casein (Table 3) has four potential sites—Ser 149, Thr 145, Thr 135, and Ser 127; but only Ser 149 is phosphorylated. Mercier et al. (34) postulated that steric hinderance due either to the conformation of κ-casein or to the presence of carbohydrate moieties on or near these hydroxy amino

TABLE 3. Selected sequences in casein polymorphs[a,b].

	Location
Sequences containing phosphate groups	
46 SerP–Glu–SerP–Thr–Glu–Asp	α_{s1}-Casein A, B, C, D
53 ThrP–Met–Glu–Asp	α_{s1}-Casein D
64 SerP–Ile–SerP–SerP–SerP–Glu–Glu–Ile	α_{s1}-Casein A, B, C, D
75 SerP–Val–Glu–Gln	α_{s1}-Casein A, B, C, D
115 SerP–Ala–Glu–Glu	α_{s1}-Casein A, B, C, D
15 SerP–Leu–SerP–SerP–SerP–Glu–Glu–Ser	β-Casein A^1, A^2, A^3, B, C
35 SerP–Glu–Glu–Gln	β-Casein A^1, A^2, A^3, B
35 SerP–Lys–Glu–Gln	β-Casein E
149 SerP–Pro–Glu–Val	κ-Casein A, B
Potential sites for phosphorylation[c]	
127 Ser–Gly–Glu–Pro	κ-Casein A, B
135 Thr–Ile–Glu–Ala	κ-Casein B
135 Thr–Thr–Glu–Ala	κ-Casein A
145 Thr–Leu–Glu–Ala	κ-Casein B
145 Thr–Leu–Glu–Asp	κ-Casein A

[a] From Bingham (6).

[b] Sequence data were obtained from the following sources: α_{s1}-casein A, B, C, and D (16, 33); β-casein A^1, A^2, A^3, B, and C (35); β-casein E (17); and κ-casein A and B (32).

[c] These are peptides containing Ser/Thr-X-Glu/SerP sequences, in which the serine is not phosphorylated.

acid residues may prevent phosphorylation of potential sites.

According to the hypothesis of the French group, it must be assumed that phosphorylation occurs in a sequential manner: Ser-X-Glu must be phosphorylated first in order to generate SerP. This idea is supported qualitatively by evidence from human caseins (15, 18); these caseins occur in multiphosphorylated forms containing zero to five phosphate groups per monomer (Fig. 4). If glutamic acid is the first specificity determinant, then Ser 9 and Ser 10 should be equally available for phosphorylation. Both serines are phosphorylated in the diphospho-casein, and these serine phosphates then may serve as specificity determinants for further phosphorylation. However, it is difficult to explain why Thr 3 is the fifth site for phosphorylation inasmuch as glutamic acid, not phosphoserine, is the specificity determinant for this site. Possibly threonine is not equivalent to serine in its ability to act as an acceptor for phosphate.

Mano and Imahori (30) have emphasized the importance of acid residues in close proximity to the serines that are phosphorylated. Reexamination of the casein sequences in Table 3 suggests that Ser-A-A* or Ser-X-A*-A might enhance the rate of phosphorylation (X is any amino acid, A is an acidic group, aspartic acid, glutamic acid, or serine phosphate, and A* is either of the latter two acids). This sequence occurs in all phosphorylated sites in the β-casein polymorphs with the exception of β-casein E, which contains lysine rather than glutamic acid at position 36 and seven out of eight sites in α_{s1}-casein, the exception being Ser 75 (Table 3). The fact that the double acids do not occur in β-lactoglobulin or κ-casein B offers a partial explanation as to why certain serines and threonines in these proteins are not phosphorylated. However, this theory explains neither the phosphorylation of Ser 149 in κ-casein A and B nor the lack of phosphate at threonine 145 in κ-casein A, which does have an adjacent double acid (Glu-Asp). The stepwise phosphorylation of human casein can be understood better by postulating the enhancement of phosphorylation by the presence of two acidic amino acid residues. This could explain, in part, why Thr 3 of human casein is the last amino acid phosphorylated because phosphoserine 6 and glutamic acid 5 are required for maximum reactivity.

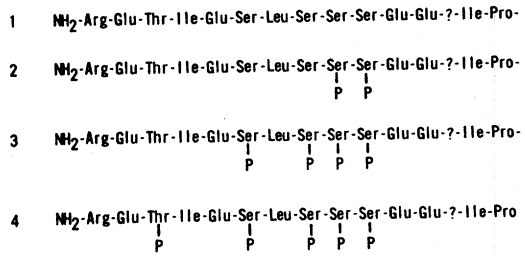

FIG. 4. The primary structure of the N-terminal end of human casein containing 0, 2, 4, and 5 phosphate groups per monomer according to Greenberg et al. (15).

While the location of phosphate groups in casein appears to be related to the neighboring amino acid sequence, the exact configuration required for phosphorylation needs further clarification. The concept that local primary structure is important in phosphorylation of proteins recently has received convincing support from Kemp et al. (20, 21), who showed the importance of an arginine residue on the N-terminal side of those serines phosphorylated by cyclic AMP-dependent protein kinases.

SUMMARY

Since 80% of milk proteins contain phosphorus, the synthesis of phosphoproteins is an important function of the lactating mammary gland. According to our present concept, the casein polypeptide chains are synthesized from amino acids on the polyribosomes of the endoplasmic reticulum. The nascent polypeptide chains enter the endomembrane lumen and are transferred to the Golgi apparatus where phosphate from ATP is added. The unique primary structure of casein makes certain serines and threonines available for phosphorylation by casein kinase. Once phosphate has been introduced into the caseins, the protein complexes (casein micelles) are formed and secreted by the Golgi vesicles. Within this general scheme, many details are lacking and should form the basis for further investigations.

Future research should be directed toward characterizing the casein kinase of the Golgi apparatus. Substrate specificity studies using model proteins and peptides should delineate

the structural and sequence requirements that promote phosphorylation.

Casein is a multiphosphorylated protein. An important question is whether the phosphate groups are equivalent. Phosphorylation could occur randomly with all serine sites equally available to the enzyme, or the phosphate groups could be added in a specific order. Although research on human caseins supports a stepwise process, there is little evidence to indicate a similar process in bovine caseins. The finding that casein kinase catalyzes the phosphorylation of dephosphorylated casein does not indicate that this enzyme is responsible for incorporating every phosphate group into casein. Therefore, several casein kinases may be needed to complete the phosphorylation of casein.

The addition of carbohydrate to κ-casein and the formation of casein micelles are intertwined with the phosphorylation since these reactions probably occur in the Golgi apparatus. Future researchers will sort out the sequence of reactions and their interrelations. Knowledge as to how milk proteins are packaged for secretion is an important and relatively unexplored area of mammary gland metabolism.

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